AN ULTRASTRUCTURAL STUDY OF LYMPHOCYTES WITH SURFACE-BOUND IMMUNOGLOBULIN*

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The interaction between molecules of antigen and the surface membrane of some lymphocytes may be the initial event that leads to their proliferation and differentiation into antibody-synthesizing cells, i.e. plasma cells. It was postulated several years ago that the site on the surface membrane which interacts or recognizes antigen should be an immunoglobulin-like molecule (Ig),[1] a hypothesis which gained support from the results of experiments by Sell and Gell using anti-Ig antibodies (1). These investigators found that lymphocytes proliferated upon treatment in vitro with anti-Ig antibody, implying that a membrane reaction had occurred between antigen and antibody (anti-Ig and Ig). It was not until very recently that Ig molecules were directly identified on the surface of lymphocytes (2–4). Using a technique by which viable lymphocytes were treated with antibodies against Ig tagged with fluorescein or radioiodine, it was possible to visualize discrete deposits of Ig on their surface. Such deposits of Ig on lymphocytes had not been detected by conventional immunohistochemical methods that employed smears of fixed cells or histological sections. Only a certain portion of the lymphocytes in the spleen and lymph nodes have detectable surface-bound Ig molecules, most of these being derived from the bursa of Fabricius in chickens (5) or the bone marrow in mammals (6). In contrast, Ig has not been demonstrated on thymus-dependent lymphocytes by current methods.

The present study was undertaken to further investigate at the ultrastructural level the presence of Ig on the surface of murine lymphocytes using electron microscopic radioautography. In addition, its purpose was to establish the distribution of Ig molecules on the cell surface and to compare the ultrastructural features of cells that possess surface Ig with those cells that lack detectable Ig.

Materials and Methods

Surface Ig was investigated on cells from the thymus glands, lymph nodes, and spleens of 10-wk old male CBA/J mice (Jackson Laboratories, Bar Harbor, Maine) using a rabbit anti-

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[1] *Abbreviations used in this paper:* Ig, immunoglobulin; KLH, keyhole limpet hemocyanin; RAMG, rabbit anti-mouse immunoglobulin.

mouse-Ig (RAMG) and electron microscopy radioautography. In brief, the cell suspensions were incubated in vitro with radioiodinated antibodies, washed, and processed for radioautography.

The RAMG was prepared by hyperimmunizing New Zealand rabbits with 2–5 mg of a gamma globulin–rich fraction of mouse sera in Freund's incomplete adjuvant once a month for about 10 months. The isolated antibody precipitated all classes of mouse Ig and mouse kappa light chains. RAMG absorbed with mouse Ig and a rabbit Ig with antibody activity against keyhole limpet hemocyanin (KLH) were used for control studies. RAMG was absorbed by passing it through a Sepharose 4B (Pharmacia Fine Chemicals Inc., Piscataway, N. J.) column that had bound mouse Ig (Ig was obtained by preparative electrophoresis of serum). Thus 5 mg of RAMG were passed through a column (10 × 0.5 cm) that contained 2 mg of Ig in about 5 ml of sedimented Sepharose. The Ig had been attached to Sepharose by treatment with cyanogen bromide (7). The antisera to KLH was obtained from rabbits hyperimmunized with KLH in complete Freund's adjuvant. The Ig fraction of all antisera was isolated by standard preparative methods involving precipitation by ammonium sulfate and diethylaminoethyl-cellulose chromatography.

The Ig fractions of all the antisera were labeled with ^{125}I by a chloramine-T procedure (8). In most experiments, 50 μg of Ig was incubated with 50 μg of chloramine-T and 6–8 mCi of Na-^{125}I in about 30-50 μl for 10 min at 4°C. The reaction was stopped by addition of 100 μg of sodium metabisulfite. Free iodine was separated from protein-bound iodine by passing the solution of protein through a Sephadex G-25 column (10 × 0.5 cm). The labeled antibodies were used within 1 hr after iodination.

The cells from the thymus, spleen, and lymph nodes were harvested aseptically, washed once with Eagle's media, and 25 × 10^6 of them were incubated with 40 μg of the antibodies labeled with ^{125}I (specific activities varied from 18 to 23 μCi/μg) at 4°C for 30 min in a total volume of 100 μl; then the cells were washed five times with media, fixed in paraformaldehyde-glutaraldehyde in 0.1 M sodium cacodylate buffer for 1 hr at room temperature (9), washed with buffer, and postfixed in 1% osmium tetroxide in distilled water for 1 hr at 4°C. After fixation, the cells were dehydrated and embedded in Epon (10). For electron microscopic radioautography, thin sections were cut and placed on bare copper grids. The grids were coated with Ilford L-4 emulsion (Ilford Ltd., Ilford, Essex, England) diluted 1:1 with distilled water and exposed at 18°C from 6 to 12 days (11). After exposure, the grids were developed in Kodak D-19 developer for 3 min, washed in distilled water, fixed in full strength Kodak acid fixer for 2 min, and again washed in distilled water. Immediately after development while still wet, the grids were stained in Reynolds' lead citrate for 5 mins (12). Observations were carried out on a Philips 200 electron microscope (Philips Electronic Instruments, Mount Vernon, N.Y.).

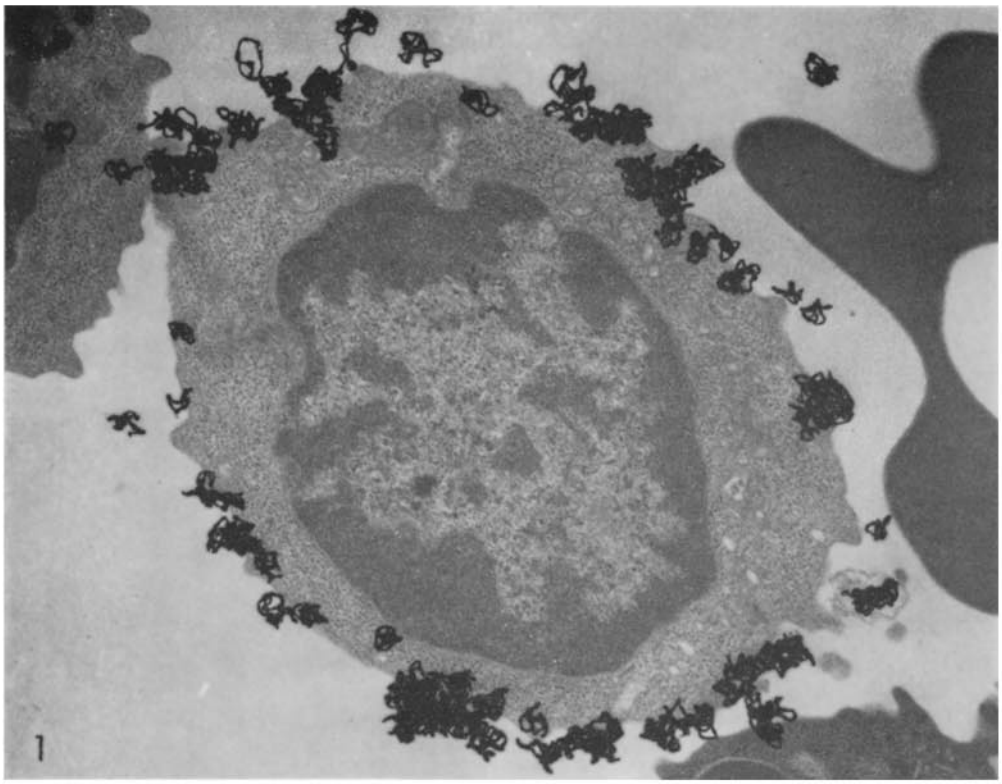
RESULTS

The majority of cells revealed no radioautographic label in control experiments in which the cells were incubated with RAMG-^{125}I absorbed with mouse Ig or with anti-KLH-^{125}I antibody. About one per every five or six cells had an isolated grain on their surface.

Some lymphocytes in the spleen and lymph nodes when incubated with RAMG-^{125}I exhibited radioautographic grains. The number of lymphocytes

FIG. 1. Electron microscopic radioautograph of a spleen lymphocyte after treatment with RAMG-^{125}I. Note random distribution of label over the cell surface. × 7500.

FIG. 2. Electron microscopic radioautograph of a spleen lymphocyte showing RAMG-^{125}I label. Label is more prominent at one pole of the cell. × 7500.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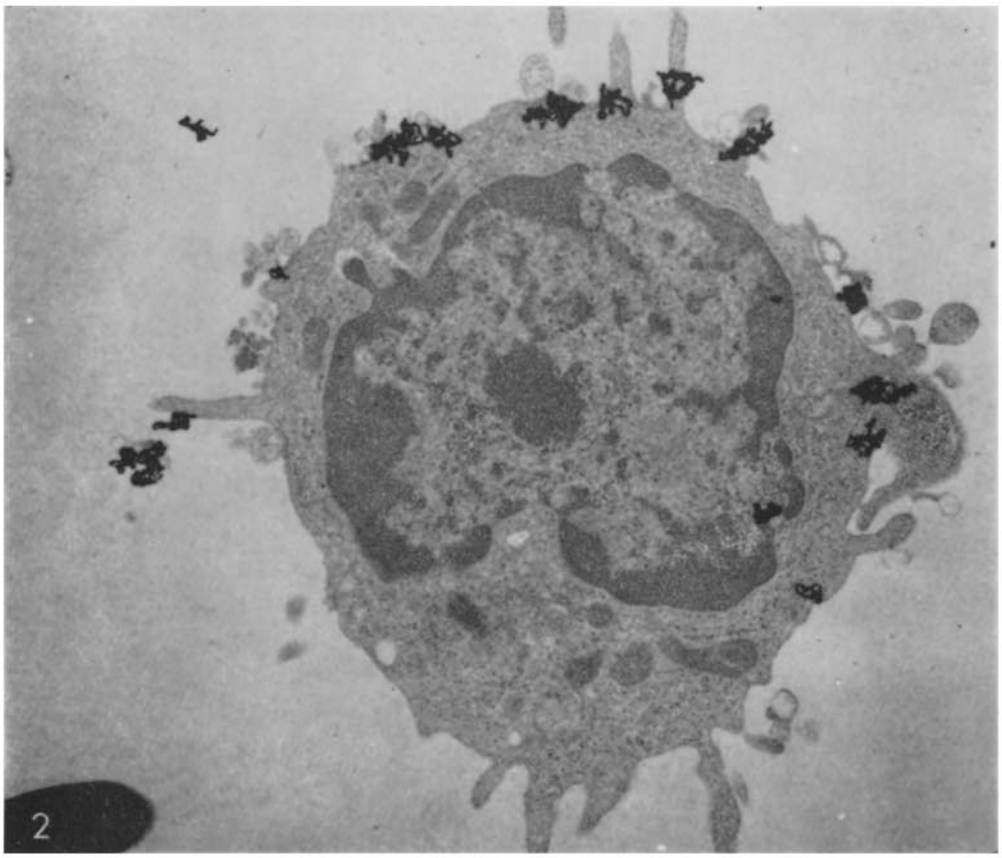
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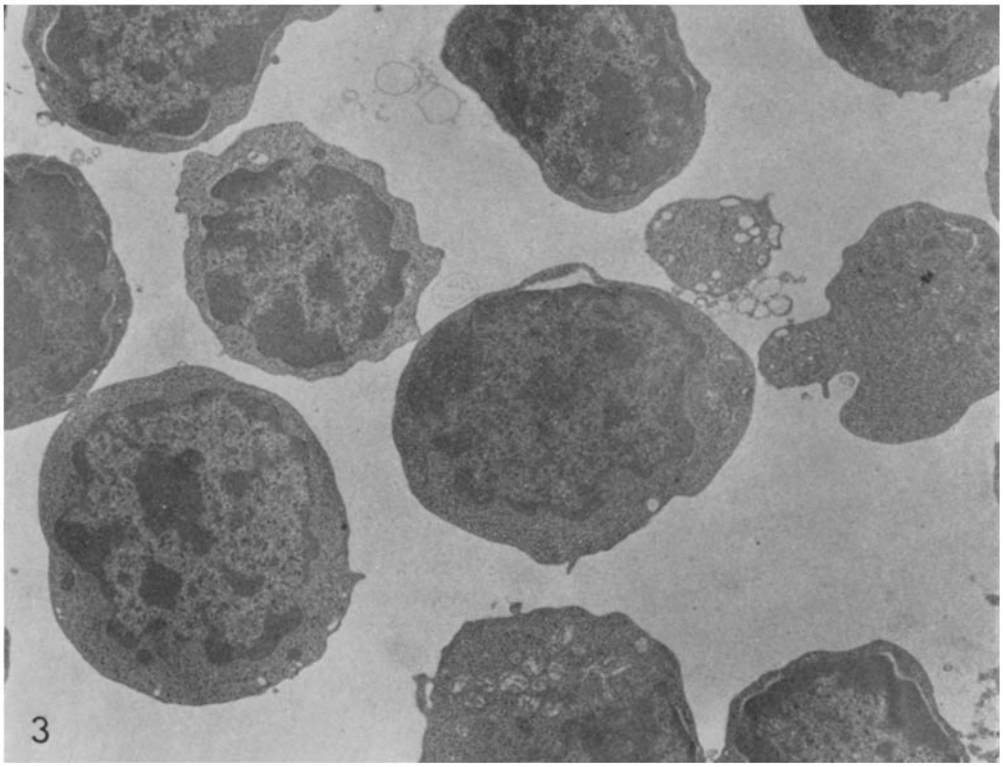


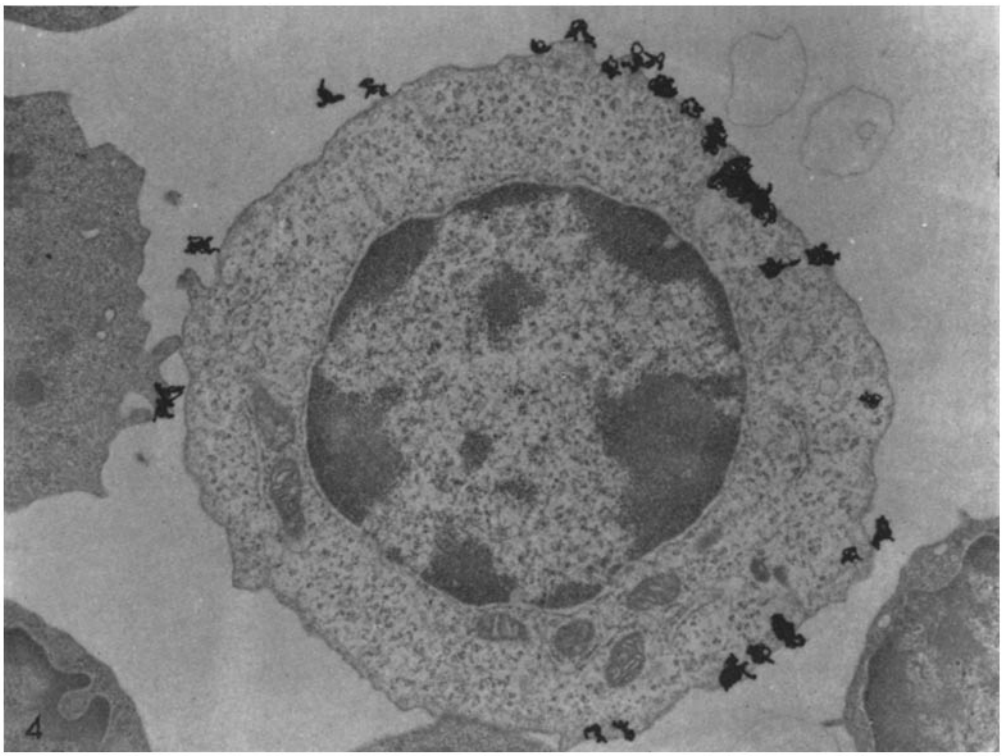
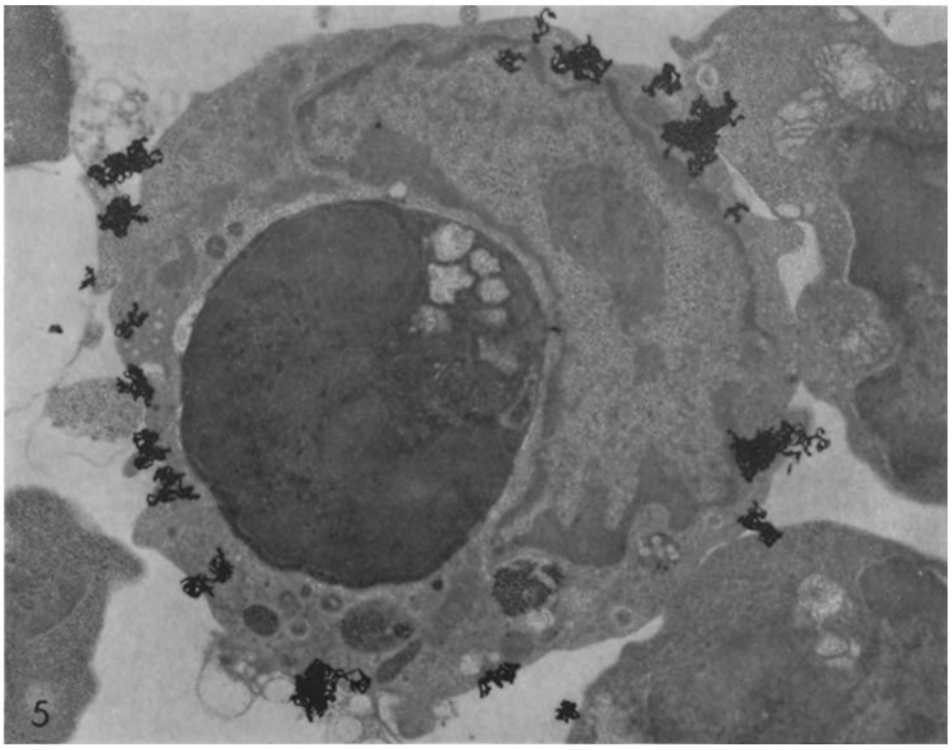


FIG. 3. Electron microscopic radioautograph of thymus lymphocytes after RAMG-^{125}I treatment. The majority of thymus lymphocytes are not labeled and in this photomicrograph no such labeled cells are included. The level of background was low in all the studies; note that in this field no background grains are observed. × 3500.

FIG. 4. Electron microscopic radioautograph of a thymus lymphocyte after treatment with RAMG-^{125}I. Cytoplasm contains fewer ribosomes than most thymus lymphocytes. Note also the lower nuclear to cytoplasmic ratio. × 10,000.



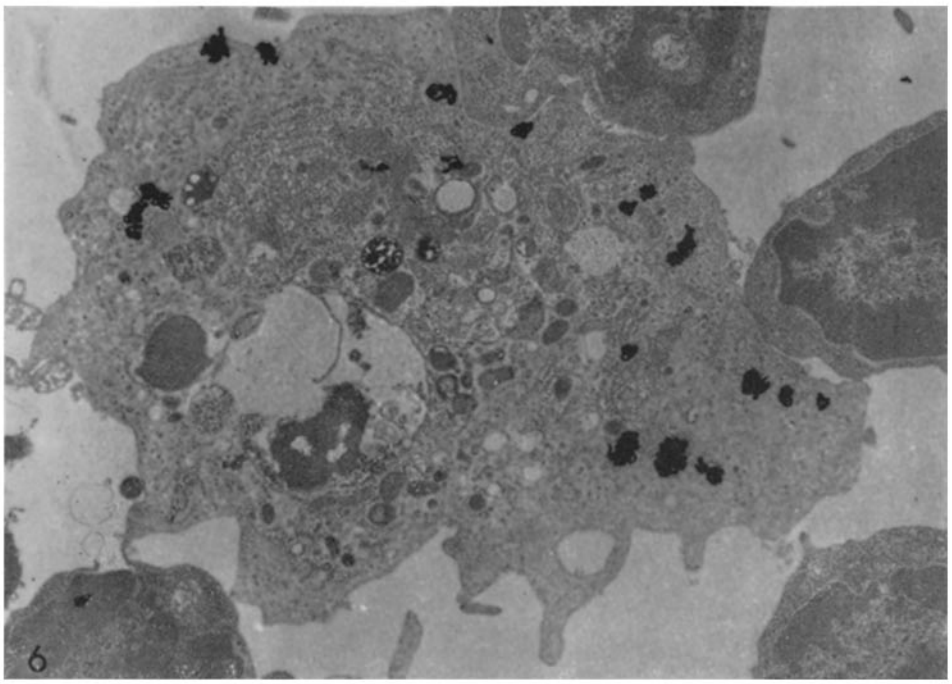


FIG. 5. Electron microscopic radioautograph of a spleen macrophage showing label on the plasma membrane after treatment with RAMG-^{125}I. Macrophage contains phagocytized lymphocyte. × 7500.

FIG. 6. Electron microscopic radioautograph of a thymus macrophage showing intracellular label after treatment with RAMG-^{125}I. The number of macrophages in the thymus was about 1/300 cells. × 7500.

that possessed grains, i.e. surface-bound Ig, ranged from 60% in spleen to 40% in lymph nodes. These figures are roughly comparable to those obtained with fluorescent antibody and light microscopic radioautographic methods (4). The label was exclusively associated with the surface membranes of the lympho-

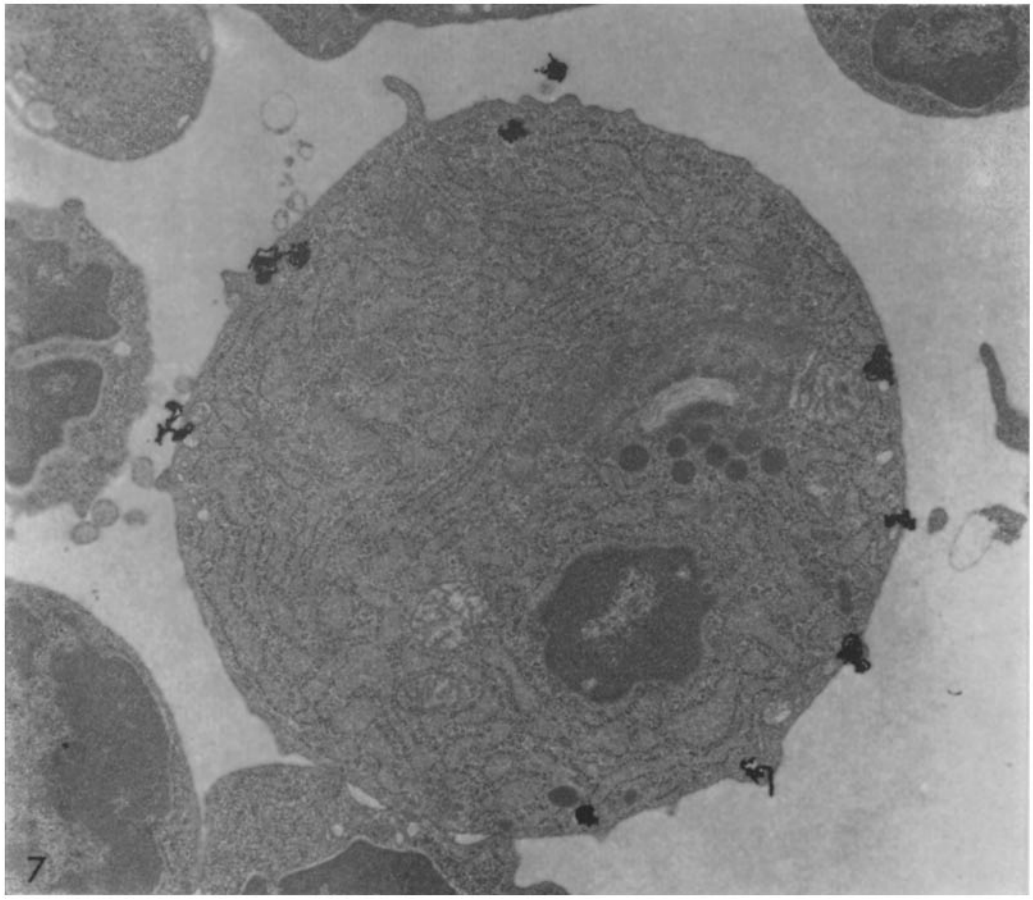


FIG. 7. Electron microscopic radioautograph of a plasma cell showing surface label after treatment with RAMG-^{125}I. Note the paucity of grains in contrast to labeled lymphocytes (cf. Fig. 1). × 7500.

cytes in a random distribution (Fig. 1). In a few examples, however, the label was found to be more prominent at one pole of the cell (Fig. 2). The mean number of grains per section of a lymphocyte was 16.3 on cells exposed to the radioautographic emulsion for 6 days; this number increased to a point where

counts could not be determined when the incubation time was extended to 12 days.

Lymphocytes with detectable surface-bound Ig had the general morphological features of typical small and medium-sized lymphocytes, i.e. marginated heterochromatin in addition to a patchy chromatin distribution throughout the nucleus, high nuclear to cytoplasm ratio, small Golgi, sparse endoplasmic reticulum, few mitochondria, occasional inclusion bodies, and numerous ribosomes either in the form of polyribosomes or monoribosomes (Fig. 1). In the lymph node and spleen, morphological differences between labeled and nonlabeled lymphocytes were not observed. Furthermore, differences were not apparent within the labeled lymphocyte population regardless of the amount and pattern of label, i.e., some cells had 15 grains on their surface while others had 25 grains but they did not appear structurally different.

In the thymus gland most of the lymphocytes did not show detectable surface-bound Ig (Fig. 3). A few lymphocytes (1/300), however, did exhibit grains and these labeled cells appeared to have unique morphological characteristics not seen in the majority of thymus, lymph node, or spleen lymphocytes. In this regard, all labeled thymic lymphocytes were observed to have a higher cytoplasmic to nuclear ratio (1:3) and the cytoplasm was less dense as evidenced by the numbers of ribosomes that were present (Fig. 4). The labeled thymus lymphocytes had fewer ribosomes per unit area when compared to nonlabeled lymphocytes. A great many of the ribosomes in the labeled thymic cells were discernible as monoribosomes.

In addition to lymphocytes, Ig was also found to be associated with macrophages and plasma cells in all three tissues examined. The label associated with the macrophages was located both on the cell surface and in intracellular vesicles (Figs. 5 and 6). The label within the cell was thought to represent endocytosed free Ig or engulfed cellular debris from degraded cells that contained bound Ig. The label for Ig on macrophages was nonspecific. The macrophages were also labeled in control experiments using RAMG absorbed with Ig or anti-KLH. Plasma cells in the thymus, spleen, and lymph nodes were also labeled with specific RAMG but the number of grains per cell section was less (mean 8) than that seen on lymphocytes (Fig. 7).

Most of the erythroid and myeloid elements in the spleen (up to 15–20% of the total cells) showed no radioautographic grains when exposed to RAMG. Furthermore, those few cells that were labeled exhibited only one or two grains.

DISCUSSION

The present ultrastructural study confirms and extends the studies at the light microscopic level and indicates that Ig molecules are present on the surface of a certain population of lymphocytes. The control studies showed that the radioautographic label was indicative of a specific reaction between labeled

anti-Ig and its antigen, i.e., Ig. Thus, cells exposed to either RAMG, which had no anti-Ig activity as a result of absorption with Ig, or to anti-KLH were essentially negative. In addition, within the cell population exposed to labeled RAMG there was an internal control in that all cells not involved in the immune reaction such as those belonging to erythroid and meyloid lines showed no radioautographic label.

In addition to the results of previously reported light microscopic studies, the present work revealed a number of new findings. First it gave an indication of the manner in which Ig molecules are distributed on the cell surface. The labeled Ig appeared to have a random distribution over the total cell surface at 4°C. This random distribution is contrary to some fluorescent microscopic findings that have shown a concentration of Ig on one pole of the cell (2, 3, 4). It has recently been shown, however, that the distribution pattern of membrane Ig-anti-Ig complexes is temperature dependent (13, 14). At 37°C there is a rapid movement of these complexes to one pole of the lymphocyte where they are internalized in vesicles. Therefore, it is imperative that a low temperature be maintained to study surface Ig topography. The finding that labeled cells contained 15–25 grains when exposed for 6 days to the emulsion indicated that each grain may not represent all Ig molecules present. It has been estimated that one lymphocyte contains about 50,000–150,000 molecules/cell (6). Assuming that a small lymphocyte is about 6 μ in diameter and that each section is about 600 A in thickness, then each section, depending upon the area of membrane which it includes, should contain an average of 500–1500 molecules. (This is assuming an equal distribution of Ig throughout the cell.) Hence, each grain observed represented 50–100 molecules of Ig. (This number could be reduced about 10 times in sections exposed for 12 days.)

A second new finding concerns the presence of very few labeled lymphocytes in the thymus. Indeed, although the majority of the thymic lymphocytes were negative for surface-bound Ig, a few lymphocytes were labeled and these had unique morphological characteristics. The origin and significance of these cells, however, remain to be determined. It can be postulated that the labeled lymphocytes may be the elusive thymic cells with Ig on their membranes or that they may be bone marrow-derived cells that have migrated into the thymus. However, it should be stressed that cells similar to the labeled thymic lymphocytes were not observed in the spleen or lymph nodes. Along these lines, labeled bone marrow-derived and unlabeled thymus-derived lymphocytes in the spleen had identical structure. Further studies are now in progress in an attempt to learn the origin of these cells. The absence of detectable Ig on thymic cells has been analyzed in a previous study (6).

A third point concerns the amount of Ig on the membrane of plasma cells, which was quite scarce. The plasma cell is an actively secreting cell that presumably results from a previous interaction of lymphocytes with antigen and which at a given time contains most of its Ig in intracytoplasmic organelles in

the process of secretion. In contrast, the "resting" lymphocytes with surface Ig have about half of their content of Ig displayed on the surface membrane (4), as would be expected if the main function of this lymphocyte-associated Ig is to bind extracellular antigen. (In reference 4, the figure for membrane and total cell Ig was obtained by a quantitative antigen-inhibition assay using Ig-^{125}I and intact or freeze-thawed lymphocytes.)

Finally, a finding that Ig was associated with macrophages was not surprising since these cells are known to have receptor sites for Ig and normally phagocytize foreign and autolyzed debris. It is of interest, therefore, that certain of the macrophages demonstrated differing labeling patterns, i.e., on the cell surface or within the cell or both. In some instances surface labeling could be due to their decreased phagocytic activity. In fact, macrophages that possessed surface Ig appeared morphologically to contain large amounts of endocytized material (labeled or unlabeled) that may have inhibited further phagocytic processes.

SUMMARY

This report is on a radioautographic study of lymphocytes exposed to ^{125}I-labeled anti-Ig in an attempt to identify surface-bound Ig molecules. The results as studied by ultrastructural radioautography confirmed the presence of surface-bound Ig on a certain population of lymphocytes. The specificity of the anti-Ig was determined by using appropriate controls that included the use of an absorbed anti-Ig and anti-hemocyanin antibody. The labeling pattern resulting from the interaction of labeled anti-Ig and Ig was found to be specifically associated with the cell surface and random in its distribution. Morphological differences were not apparent between labeled and nonlabeled lymphocytes in the spleen and lymph nodes. In the thymus, most lymphocytes did not exhibit detectable Ig. The few thymic lymphocytes that were labeled had unique morphological characteristics that included fewer ribosomes, many of which were monoribosomes. Relative to the amount in their cytoplasmic organelles, plasma cells had surface Ig but to a lesser degree than lymphocytes. Finally, macrophages were nonspecifically labeled and contained antibody on their membranes as well as intracellularly.

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BIBLIOGRAPHY

1. Sell, S., and P. G. H. Gell. 1965. Studies on rabbit lymphocytes in vitro. I. Stimulation of blast transformation with an antiallotype serum. *J. Exp. Med.* **122**:423.
2. Raff, M. D., M. Sternberg, and R. B. Taylor. 1970. Ig determinants on the surface of mouse lymphoid cells. *Nature (London)*. **225**:553.
3. Pernis, B., L. Forni, and L. Amante. 1970. Immunoglobulin spots on the surface of rabbit lymphocytes. *J. Exp. Med.* **132**:1001.

4. Rabellino, E., S. Colon, H. M. Grey, and E. R. Unanue. 1970. Immunoglobulins on the surface of lymphocytes. I. Distribution and quantitation. *J. Exp. Med.* **133:**156.
5. Rabellino, E., and H. M. Grey. 1971. Immunoglobulins on the surface of lymphocytes. III. Bursal origin of surface immunoglobulins on chicken lymphocytes. *J. Immunol.* **106:**1418.
6. Unanue, E. R., H. M. Grey, E. Rabellino, P. Campbell, and J. Schmidtke. 1971. Immunoglobulins on the surface of lymphocytes. II. The bone marrow as the main source of lymphocytes with detectable surface-bound immunoglobulins. *J. Exp. Med.* **133:**1188.
7. Cuatrecasas, P., M. Wilcheck, and C. P. Anfinsen. 1968. Selective enzyme purification by affinity chromatography. *Proc. Nat. Acad. Sci. U.S.A.* **61:**636.
8. Greenwood, F. C., W. M. Hunter, and J. S. Glover. 1963. The preparation of I^{131} labeled human growth hormone of high specific radioactivity. *Biochem. J.* **89:**114.
9. Karnovsky, M. J. 1965. A formaldehyde-glutraldehyde fixative of high osmolarity for use in electron microscopy. *J. Cell Biol.* **27:**137A. (Abstr.)
10. Luft, J. H. 1961. Improvements in epoxy resin embedding methods. *J. Biophys. Biochem. Cytol.* **9:**409.
11. Caro, L. G., and R. P. Van Tubergen. 1962. High resolution autoradiography. I. Methods. *J. Cell Biol.* **15:**173.
12. Reynolds, E. S. 1963. The use of lead citrate at high pH as an electron opaque stain in electron microscopy. *J. Cell Biol.* **17:**208.
13. Taylor, R. B., W. P. H. Duffers, M. C. Raff, and S. de Petris. 1971. Redistribution and sinocytosis of lymphocyte surface immunoglobulin molecules induced by anti-immunoglobulin antibody. *Nature (New Biol.) (London).* **233:** 225.
14. Unanue, E. R., W. D. Perkins, and M. J. Karnovsky. 1972. Endocytosis by lymphocytes of complexes of anti-Ig with membrane bound Ig. *J. Immunol.* **108.** In press.